TERMINATION OF PUPAL DIAPAUSE IN *CALLOPHRYS SHERIDANII* (LYCAENIDAE)

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ABSTRACT. When *Callophrys sheridanii neoperplexa/newcomeri* pupae were incubated at 4°C for 100 days, 65% of the pupae eclosed within 8 days and the remaining 35% eclosed gradually 45 to 200 days after the termination of the chilling. Non-chilled pupae stopped their development at the eye pigmentation stage. More than 60% of adult eclosion was observed within 30 min after lights on. These results suggest that adult eclosion of this species occurs abruptly in the early morning on the first few warm days in spring, and one of the factors explaining the sporadic late records in the field is the gradual termination of the pupal diapause.

Additional key words: circadian rhythm, eclosion, low temperature, endocrinology.

Insect development is governed primarily by ecdysteroids and juvenile hormone (JH). Prothoracicotropic hormone (PTTH) from brain neurosecretory cells stimulates prothoracic glands to secrete 3-dehydroecdysone in *Manduca sexta* L. (Sphingidae) (Warren et al. 1988), it is then converted to ecdysone and then to an active hormone, 20-hydroxyecdysone, which initiates development (Rees 1989). Ecdysteroids initiate the larval molt in the presence of JH secreted from corpora allata. In the last instar larva in Lepidoptera, a small peak of ecdysteroids in the absence of JH causes the cessation of feeding, larval-pupal commitment of the epidermis, and the onset of wandering behavior. Subsequent exposure to ecdysteroids initiates pupation in the presence of JH (Riddiford & Hiruma 1990). This JH in the wandering stage is important to coordinate PTTH release (Hiruma 1986) and subsequently it stimulates prothoracic glands to secrete ecdysteroids that are responsible for pupation, in addition it prevents to become an adultoid (a pupa with some adult structures) in some lepidopteran species (see Hiruma 1980). Adult development is caused by an ecdysteroid surge in the pupal stage in the absence of JH (see Riddiford & Hiruma 1990 and Nijhout 1994 for reviews).

Pupal diapause is characterized by the failure of the ecdysteroid secretion from prothoracic glands after pupation, which is primarily due to the failure of the release of PTTH from brains (Williams 1946, 1947, Denlinger 1985). The brain of a diapausing pupa can be activated to secrete PTTH by chilling, thus terminating diapause so that adult development is initiated (Williams 1952).

Callophrys sheridanii Edwards (Lycaenidae) is widely distributed in the western U.S., and differentiated to several different subspecies (Scott 1986). In Washington State, there are two subspecies distinguished: *C. s. neoperplexa* Barnes and Benjamin occurring in the Columbia Basin and adjoining regions, and *C. s. newcomeri* Clench occurring in the south, southeast, and the east slope of the Cascade Mountains. The two intergrade in the east central Cascades, from lowland steppe to high mountains in Kittitas and Chelan Counties west of the Columbia River (Pelham & Hiruma, pers. obs.). This species is considered univoltine and enters obligatory diapause in the pupal stage for hibernation (Scott 1986).

In this paper, we show that diapause intensity in *C. s. neoperplexa/ newcomeri* pupae is heterogeneous and the timing of the adult eclosion responds to photoperiod. Also, we discuss the relationship between the results obtained in the laboratory and those of field observations.

MATERIALS AND METHODS

Eggs and first instar larvae of *Callophrys sheridanii neoperplexa/newcomeri* were collected from *Eriogonum compositum* Dougl. (var. *leianthum* Hook.) (Polygonaceae) in Schnebly Coulee, Kittitas County, Washington, and some of the eggs were laid in the laboratory on *E. compositum* by females caught in the same location. All the field caught eggs and larvae were brought into the laboratory, and reared under crowded conditions on *E. compositum* leaves in a plastic dish (14 cm diameter/2 cm height) at 26°C in a 17L:7D photoperiod. Twenty to 30 larvae were reared together until third instar larvae, then reduced the numbers to 10 to 15 in the fourth instar larvae. The leaves were changed daily. The food plant was wrapped with plastic bags and kept at 4°C no longer than 2 weeks. Lights off, the beginning of a new day, was set at 00:00 AZT (Arbitrary Zeitgeber Time) (Pittendrigh 1965). In this condition, cannibalism in larvae of this species was not observed as observed in the closely related European species, *Callophrys rubi* L. (Lycaenidae) (Ford 1945).

Pupae were kept at 26°C in a 17L:7D photoperiod for 67 to 77 days after pupation, they were then transferred to a 4°C incubator for 100 days at dark, followed by transferring back to the 26°C condition (17L:7D). Non-chilled pupae were kept at 26°C at dark for 100 days instead of placing at 4°C, then at 26°C in a 17L:7D condition. Adult eclo-
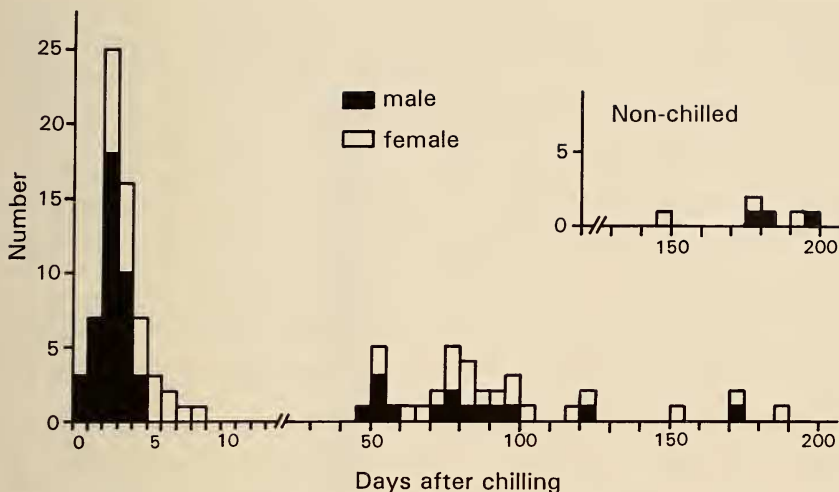
FIG. 1. Adult eclosion from *Callophrys sheridanii neoperplexa/newcomeri* pupae. Pupae were kept at 26°C for 67 to 77 days after pupation, they were then transferred to 26°C condition 100 days after the incubation at 4°C (n = 122). One male eclosed and 2 pupae died at 4°C. Nineteen pupae died after the chilling, but 15 developed to adult inside of the pupal cuticles and died without eclosion, probably because of desiccation. As a control, pupae (n = 50) were kept at 26°C throughout the experiments (see Materials and Methods), and the days of eclosion were calculated as 0 at the time of the termination of the chilling in the experimental (inset). Only 6 eclosed, and one developed to adult but did not emerge ("150 days" is equivalent to "327 days after pupation"). The rest of them died by desiccation without forming any scales. Dead pupae were excluded from the data.

sion was checked daily for about 400 days after pupation, and dissected all the non-eclosed pupae to examine whether or not they died.

RESULTS

Time of eclosion of diapausing pupae. All pupae developed to the stage to which compound eye pigmentation occurred within 67 to 77 days at 26°C in a 17L:7D photoperiod, then stopped their development (n = 202). Most of the pupae produced very audible creaking sounds when disturbed as reported in many lycaenid pupae (Downey 1966, Brakefield et al. 1992), which was predicted by the presence of the stridulating organ in *C. sheridanii* (Downey 1966). The sound was produced from shortly after pupation to the beginning of the eye pigmentation stage.

Figure 1 shows that if pupae were not exposed to 4°C, only 12% of the pupae eclosed (6 out of 50) 326 to 377 days after pupation, which were equal to 149 to 200 days calculated as days after chilling (see below). When pupae were placed at 4°C for 100 days, the adult eclosion

occurred in 65% of the pupae within 8 days after the termination of the chilling. One male eclosed even at 4°C. The remaining 35% eclosed gradually over about 200 days after chilling (Fig. 1). Apparently, the pupal diapause was terminated by the incubation at 4°C, and the diapause intensity of this population is not homogenous. This pattern of adult emergency indicates that there are at least two physiologically different pupae in the population. One is very sensitive to a low temperature to break diapause and adult eclosion occurs within 10 days after the termination of chilling (group 1), and the other is much less sensitive to a low temperature whose adult eclosion occurs 50 to 100 days after the termination of chilling (group 2). Since non-chilled pupae did not emerge more than 140 days (Fig. 1), the adult eclosion of the group 2 pupae is considered to have responded to the chilling.

Sex ratio. Sixty-four percent (42 of 66) of adults were male in the group 1, but only 39% (16 of 41) in the other groups (Fig. 1). Male ratio was statistically higher in group 1 (P ≪ 0.001), but female ratio was statistically higher in the others (P < 0.003). The overall sex ratio (male/male + female) of the adults in the laboratory reared population was 0.542 and it was not significantly different at P > 0.05 (n = 107). According to the records deposited in the Burke Memorial Museum of the University of Washington and those of our private collection, males were more commonly caught than females in the gulch bottom of Schnebly Coulee, where the food plant occurs, in mid March to mid April, and the sex ratio was 0.77 (n = 188) (significantly different at P ≪ 0.001).

Timing of adult eclosion. Of the adults that emerged 1 to 5 days after chilling, more than 60% did so within 30 min after lights on (Fig. 2). This indicates that adult eclosion most likely occurs in the early morning in the field.

DISCUSSION

The duration of diapause under well-defined environmental conditions usually is quite consistent for a given population, and the insect's capacity to respond to environmental cues can also be directed by its genetic potential, sex, food plants and maternal history (Denlinger 1985, Pratt & Ballmer 1993). Diapause termination is linked directly to a specific environmental cues, and the exposure to a low temperature was one of the main factors in *C. s. neoperplexa/newcomeri* (Fig. 1). Yet the trait is quite polymorphic for the termination as found in *Hyalophora cecropia* L. (Saturniidae) (Waldbauer & Sternburg 1973) whose bimodality for the termination of the pupal diapause has a genetic basis. Figure 1 suggests that the population of *C. s. neoperplexa/newcomeri* is genetically heterogeneous, which also is evidenced by the mixed degrees of the postmedian line. The similar bimodal adult emergence was
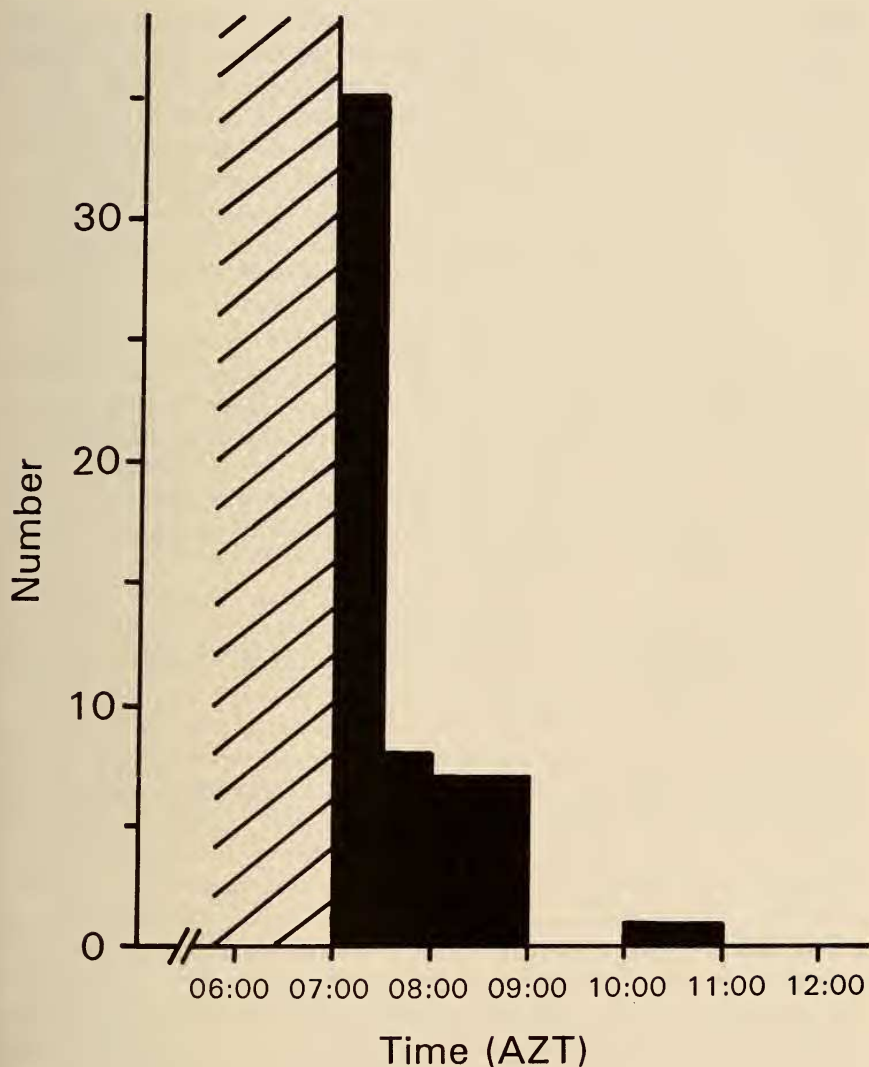
FIG. 2. Distribution of adult eclosion for *C. s. neoperplexa/newcomeri* under 17L:7D. Observations were performed on adults eclosed 1 to 5 days after the termination of chilling (n = 58). Seven adults eclosed during the dark.

also observed in the laboratory reared *Incisalia mossii mossii* Edwards (Lycaenidae) (Hiruma, unpubl. data).

The developmental stage of the arrest of diapausing pupae varies among species. In *M. sexta* (Bell et al. 1975, Bowen et al. 1984) and *H. cecropia* (Williams 1946), pupae enter diapause shortly after pupation

due to the inactivation of the brains, whereas in *Luehdorfia japonica* Leech (Papilionidae), an univoltine species, the pupae complete almost all adult development by the beginning of winter. Yet adult eclosion does not occur until the following spring (Hidaka et al. 1971). It is interesting that pupal development occurs up to a certain level before the onset of diapause in *C. s. neoperplexa/newcomeri*. Probably, the brain-prothoracic gland axis is still active to secrete ecdysteroid even after pupation, so that the pupal development occurs until eye pigmentation stage. The determination of PTTH and/or ecdysteroid titer in the hemolymph is necessary to ascertain this hypothesis.

Adult eclosion occurs at specific times of day in *M. sexta, Antheraea pernyi* Guérin-Ménéville (Saturniidae) and *H. cecropia*; it is controlled by a circadian clock and has been classified as a "gated" event (Truman 1985). For the gated adult eclosion in *M. sexta*, the brain contains the clock that determines when eclosion hormone release will occur. The adult eclosion of *C. s. neoperplexa/newcomeri* also is thought to be controlled by a biological clock, and this may be due to the release of eclosion hormone shortly after lights on.

According to our results reported in this paper, more than a half of the pupal population of *C. s. neoperplexa/newcomeri* at a given location must emerge in the early morning on the first few warm days in Spring, followed by sporadic emergence throughout summer. Our field observation partially supports this hypothesis. Adults of this species fly in early March to mid April in the lowland steppe such as Schnebly Coulee (500 m), but the peak of the flying period of fresh adults in each year is usually 7 to 10 days (Hiruma & Pelham, pers. obs.), which is supported by the outbreak of the eclosion of the diapausing pupae shortly after the termination of chilling (Fig. 1). In the higher elevations such as Chumstick Mountains (1600–1700 m) in Chelan County, Washington, and Reecer Creek Road (1400 m) in Kittitas County, Washington, adults appear in early April to early May (depending on the snow melt), but there are sporadic late records in both locations. In Schnebly Coulee and its vicinity, a relatively fresh female specimen was caught on 7 May 1988, two males on 16 May 1987 and a male on 20 May 1984 (we did not find any adults in mid April in these years), and in the Chumstick Mountains, a fresh female on 16 July 1991 (Hiruma & Pelham, pers. obs.). Similar late records have been reported many times in *C. rubi*, and it is debatable whether or not these late records are due to second generations (see Ebert & Rennwald 1991 for detailed discussions). Based on our results, these late records are most likely due to the gradual eclosion depending on the diapause intensity, although we cannot rule out the possibility of second generations.

Sexual differences in diapause response is common (see Denlinger

1985 for examples), and *C. s. neoperplexa/newcomeri* seems to exhibit this feature. Males were more common than females in the field, which may be due not only to collecting efficiency, but also to fewer eclosions of females in spring. It is well known that a certain percentage of diapausing pupae in some species of *Anthocharis* (Pieridae) and *Atrophaneura* (Papilionidae) do not emerge in the expected year after hibernation, but emerge in the same season 2 to 3 years later (Kawazoé & Wakabayashi 1976). It has been reported as an extreme example in *Eriogaster lanestris* L. (Lasiocampidae) that the adult eclosion of 15 pupae occurred 14 years after pupation (Van-Nuvel 1976), although a 7-year-diapausing period was frequently observed in this species (South 1907, Van-Nuvel 1976). This seasonal adaptation has a number of advantages for unexpected unfavorable environments. In *C. s. neoperplexa/newcomeri*, the genetic program may not be completed to do so, and as a result it causes gradual adult eclosion during the same year.

These results indicate that results obtained in the laboratory are able to explain some of the unsolved observations in the filed, and therefore both types of research will help to analyze ambiguous results.

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